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Increased number of tissue factor protein expressing thrombocytes in canine idiopathic immune mediated hemolytic anemia

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ABSTRACT

Dogs suffering from canine idiopathic immune mediated hemolytic anemia (cIIMHA) are at great risk of dying particularly in the first two weeks after the diagnosis is made. This high mortality risk may be associated with the development of thromboembolism (TE) and/or disseminated intravascular coagulation (DIC) resulting in organ failure. The exact mechanism of the development of TE and/or DIC in cIIMHA is still undetermined. Therefore, this study investigates the presence of tissue factor (TF) in thrombocytes of dogs suffering from cIIMHA, using *OptiPrep*™ for the isolation of blood cells and immunocytochemistry (ICC) to visualize TF on thrombocytes. The normalised TF quantity, acquired with 'colour deconvolution' (*ImageJ* plug in), revealed that in cIIMHA dogs the fraction TF positive thrombocytes was statistically significant higher ($P < 0.001$; mean 0.79; $n = 7$) compared to the fraction TF positive thrombocytes of the healthy dogs (mean 0.43; $n = 9$). We further have indications that the fraction of TF positive thrombocytes decreases with time and therapy, but that the progression rate differs individually. Since cIIMHA dogs have more thrombocytes that are TF-positive compared to healthy dogs, this may explain the increased risk to develop TE and DIC. Furthermore, it seems that the number of TF-positive thrombocytes in cIIMHA dogs remains high during the first two weeks of the disease, the time when the animals are at greatest health risk.

1. Introduction

Dogs suffering from cIIMHA are at great risk of dying particularly in the first two weeks after the diagnosis is made (Mueller et al., 2004). This high mortality risk may be associated with the development of TE (Carr et al., 2002; Fenty et al., 2011; Klein et al., 1989; McManus and Craig, 2001; Scott-Moncrieff et al., 2001) and/or DIC (Carr et al., 2002) resulting in organ failure. The exact mechanism of the development of TE and/or DIC in cIIMHA is still undetermined.

One explanation may be the presence of hypercoagulable thrombocytes. Indeed, there are some studies which assume that dogs suffering from cIIMHA have hypercoagulable thrombocytes, but the physiological explanation for this is still unclear (Fenty et al., 2011; Scott-Moncrieff et al., 2001; Weiss and Brazzell, 2006). Those thrombocytes may circulate in an activated state, which is determined by an increase of P-selectin expression on the cell surface (Weiss and Brazzell, 2006). After thrombocyte activation, P-selectin functions as a cell adhesion molecule and plays a role in thrombus formation by promoting thrombocyte recruitment and aggregation.

Another explanation may be that blood cells of dogs with cIIMHA express TF (Piek et al., 2011). TF is an important coagulation factor of the extrinsic coagulation pathway and is activated after cell damage. The high prevalence of monocytosis and the fact that monocytes are known TF sources, suggest that monocytes are the source of TF expression in dogs with cIIMHA (Piek et al., 2011). Indeed, some human and mouse studies demonstrated that neutrophils and monocytes produce TF (Mälarstig and Siegbahn, 2007; Maugeri et al., 2006; Rivers et al., 1975; Setty et al., 2012) and even donate it directly to thrombocytes (Giesen et al., 1999; Rauch et al., 2000; Sovershaev et al., 2012), or indirectly by microvesicles (Falati et al., 2003). However, this hypothesis, TF mRNA production by monocytes in dogs suffering from cIIMHA, has not been confirmed yet (Piek et al., 2011). The mRNA levels of IL-8, a monocyte derived chemotaxin, which normally triggers TF release by monocytes, was even significantly lower in cIIMHA dogs compared to the other hypercoagulable patient-groups (Piek et al., 2011).

An alternative explanation may be that the thrombocytes contribute to the high intravascular TF in cIIMHA, because a statistically significant relation was found between intravascular TF and both mean platelet volume (MPV) and mean platelet mass (MPM) (Piek et al., 2011). This suggests thrombocyte activation and therefore possibly TF release in cIIMHA dogs. Contrary to the scarce literature about

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veterinary research on canine thrombocyte associated TF production, numerous human studies are present providing arguments to believe that thrombocytes might be the TF source in cIIMHA patients. Some studies demonstrate that TF protein is bound to the thrombocyte cell membrane (Brambilla et al., 2008; Camera et al., 2003; Camera et al., 2016; Müller et al., 2003; Panes et al., 2007; Siddiqui et al., 2002; Vignoli et al., 2013; Zillmann et al., 2001), whereas other studies claim that resting thrombocytes are thought to contain TF protein (*i.e.* α-granules and in the open canicular system) (Müller et al., 2003) and that activated thrombocytes are capable of releasing functionally active TF protein (Siddiqui et al., 2002). If so, this could explain the increased risk of developing TE and DIC in cIIMHA.

Previously it was shown that dogs with cIIMHA have increased intravascular TF mRNA (Piek et al., 2011). In this study the objective was to gain information, by use of ICC, about TF protein expression by thrombocyte membranes of dogs suffering from immune mediated hemolytic anemia.

2. Material and methods

2.1. Patient selection

The cIIMHA dogs were referred to the intensive care unit of Department of Clinical Sciences of Companion Animals Utrecht University, between October 2014 and May 2015. Inclusion criteria for cIIMHA dogs were as follows: the Ht is < 0.30 L/L, and at least two of the following: a positive Coombs test (titre ≥ 16), spherocytosis, increased osmotic red cell fragility (Piek et al., 2012). In addition no underlying cause of the cIIMHA has been found. Inclusion criteria for the healthy dogs were: the dogs never had been in areas endemic for babesiosis, leishmaniosis or ehrlichiosis, did not receive any medication or vaccination in the last month and had no clinical disease in the 6 months previously to the study. The blood collection procedure was approved by and performed according to the standards of the Ethical Committee of Animal Experimentation of Utrecht University. A written consent was signed by the owner.

2.2. Blood collection

EDTA anticoagulated surplus blood samples (1–3 mL) were obtained by jugular venipuncture. Initial blood samples were taken prior to blood transfusion (packed red blood cells). In order to prevent excessive thrombocyte activation, samples were processed within 4 h after blood collection (Moritz et al., 2003). Blood collection of the cIIMHA dogs was performed on all hospitalization days and at follow-up appointments in the clinic. Blood was collected from healthy dogs at the ambulatory clinic.

2.3. Thrombocyte parameters

Of all blood samples a complete blood count (CBC) was made by use of a hematology analyzer (ADVIA 2120i Hematology Analyzer, Siemens healthcare), in order to measure the MPV, MPM and mean platelet concentration (MPC). In order to prevent excessive thrombocyte activation, blood samples were processed within 4 h after blood collection (Moritz et al., 2003).

2.4. Experimental design

Thrombocytes of two groups: cIIMHA (n = 7) and healthy dogs (n = 9), were compared with respect to TF protein expression by thrombocytes. In order to reproduce serial measurements, four out of seven cIIMHA dogs were followed for more than 4 consecutive days for the duration of hospitalization and follow-up appointments.

2.5. Isolation of cells for immunocytochemistry

For the study of the presence of TF in thrombocytes, neutrophils and monocytes (Mälarstig and Siegbahn, 2007; Maugeri et al., 2006; Rivers et al., 1975; Setty et al., 2012) were used as positive controls and erythrocytes as negative controls. Erythrocytes are known to be free of TF expression and/or to contain TF protein.

2.5.1. Thrombocyte isolation

Thrombocyte isolation was performed by using OptiPrep™ (Sigma-Aldrich) described by *Graham. 2002* (Graham, 2002a; Trichler et al., 2013). This procedure separates the blood sample into four distinct layers, of which the second cloudy layer containing the thrombocytes that was collected by pipette and transferred to an 1.5 mL Eppendorf tube for further analysis (ADVIA 2120i Hematology Analyzer, Siemens healthcare) to enumerate thrombocyte fractions and assess purity of the thrombocyte fractions.

2.5.2. Leukocyte isolation

By use of OptiPrep™ (Sigma-Aldrich) leukocyte rich samples were prepared (Graham, 2002b). Two different density barriers were made. A density barrier of 1.090 g/mL was used to isolate polymorphonuclear leukocytes. Monocytes were isolated with use of a density barrier of 1.077 g/mL. After centrifugation two different layers were present. One upper layer which contains the monocytes and one lower layer which contains the polymorphonuclear leukocytes. Both layers were harvested by use of a pipette and pooled into a 1.5 mL Eppendorf tube.

By use of the hematology analyzer (ADVIA 2120i Hematology Analyzer, Siemens healthcare) it was shown that the pooled pilot sample indeed contained neutrophils (2.74 × 10e9/L) and monocytes (0.40 × 10e9/L), but also thrombocytes (99 × 10e9/L). Analysis of a sample smear from the leukocyte rich sample confirmed the presence of neutrophils and monocytes, and some thrombocytes (data not published). The reason for the thrombocyte contamination is probably caused by the adjacent density barriers (monocyte: 1.077 versus thrombocyte: 1.063 g/mL) and therefore some larger thrombocytes were trapped within the monocytic layer.

2.5.3. Erythrocyte rich samples

To obtain erythrocyte rich samples the erythrocyte pellet was used from the centrifuged EDTA blood sample used for leucocyte isolation (since the leukocytes are not present in the pellet anymore, contrary to the pellet in thrombocyte isolated samples). The pellet was a highly concentrated erythrocyte sample and therefore it was diluted with PBS to obtain a suitable suspension for the cytospin for the ICC procedure.

2.6. Immunocytochemistry

From each sample type (*i.e.* thrombocytes, leukocytes or erythrocytes) a series of four cytospin slides were produced with a cytospin (Shandon Cytospin® 4, Thermo Fisher Scientific) using 50 µL sample per slide and centrifuging at 48 g for 10 min. One slide was stained with May Grünwald Giemsa (MGG) stain to check cell integrity and cell distribution. The three other slides were prepared for the ICC procedure. Cytospins were made 3–4 days before performing the ICC procedure, air dried and stored at RT.

When starting the ICC procedure, the slides were fixated in warm (RT) acetone for 3 min. The slides were air dried for about 10 min and thereafter washed 2 × 2 min with PBS-Tween (100 mL *10 x PBS* + 900 mL milliQ + 1 mL *Tween 20* (0.1%); pH 7.4).

Endogenous peroxidase activity was blocked by adding an endogenous enzyme block (Dako Denmark A/S) and the slides were incubated for 10 min at RT. After this blocking step the slides were again washed with PBS-Tween for 3 × 5 min. To prevent high background staining the slides were incubated with normal goat serum (1:10 PBS) for 1 h at RT. Following this step the three serial cytospin slides were

Table 1
Clinical signs, blood results (CBC), packed red cell blood transfusion numbers and follow up period for all cIIMHA dogs at the first hospitalization day. Highest leukocyte numbers during the hospitalization period are shown in parentheses. A − G are all 7 cIIMHA dogs. MD missing data; − no; + yes.

	Dog	A	B	C	D	E	F	G	*unit*	*reference range*
Clinical signs										
Macroscopic hemoglobinemia and/or hemoglobinuria		+	+	+	–	+	–	+		
Icteric sclera and/or plasma		+	+	+	+	+	–	+		
Blood results										
Hematocrit		0.14	0.11	0.08	0.17	0.09	0.10	0.09	l/l	0.42–0.61
Leukocytes (highest value during hospitalization)		22.4 (64.8)	49.3 (99.9)	43.3 (46.6)	32 (65.8)	34.8	29.8	57.5 (57.8)	10^9/l	4.5–14.6
Bands		2.4	4.4	6.5	0.3	MD	0.3	21.8	10^9/l	0–0.3
Reticulocytes		209.0	510.6	128.6	256.4	121.5	438.6	408.7	10^9/L	8.4–129.3
Thrombocytes		118	180	89	425	103	473	347	10^9/l	143.3–400
Spherocytosis		yes	yes	yes	yes	no	no	yes		
Test results										
Positive Coombs test		+	+	+	+	+	+	+		
Increased osmotic red cell fragility		+	+	–	+	+	–	–		
Other										
Transfusion numbers		4	2	2	2	2	1	1		
Follow up period		1.5	1.5	1.5	1.5	1.5	1.5	1.5	years	

differently treated. One slide was incubated with the primary antibody (1:100 rabbit anti-human TF antibody; monoclonal IgG; ab151748, Abcam plc) and served as test. By antibody titration from 1:500 to 1:100, the dilution of 1:100 was determined to be optimal. Another slide was incubated with the isotype antibody (1:100 anti-rabbit; monoclonal IgG; ab172730, Abcam plc) to check the specificity of the anti-TF antibody to TF. A third slide was incubated with only the primary antibody diluent (Dako Denmark A/S) and served as an internal control of the whole ICC procedure. All slides were thereafter incubated over night at 4 °C.

After overnight incubation the slides were rinsed in PBS-Tween for 3 × 5 min. All slides were separately washed in order to avoid contamination of the remaining unbound antibodies. The secondary goat anti-rabbit antibody (Dako Denmark A/S) was then added to all slides and incubated for 45 min at RT. Thereafter a next wash with PBS for 3 × 5 min followed. In order to visualize antibody binding, all slides were incubated with 3,3′-diaminobenzidine (DAB, Dako Denmark A/S) (1 mL buffer + 1 droplet DAB) for 5 min. The slides were washed again for 3 × 5 min with milliQ. To counterstain the cells, in order to visualize the cell contours, each slide was stained for 10 s with hematoxylin (HE, Dako Denmark A/S) and thereafter rinsed in tap water for 10 min.

Slides were prepared for long term storage by performing a five-step-series of dehydration by increasing the alcohol percentage from 60% to 96%. Each dehydration step took 5 min. Another two treatments included Xylene I and Xylene II and took 2 × 3 min. In order to store the slides for long term, the slides were covered with cover slips by use of vecta mount (Vector Laboratories, Burlingame, CA, USA).

From all slides microscopic photos were taken at 250 x magnification (Bresser MikroCam 9.0 MegaPixel, Bresser GmbH). To quantify TF presence, specific software was used (ImageJ, plugin 'colour deconvolution', Rasband). A HE-staining was used to visualize the cell contours. 'Colour deconvolution' plug in, which per photo separates the DAB staining from HE staining, enables calculation of DAB and HE stained cell areas. The formula to normalise TF quantity was as follows: fraction of TF positive cell area = DAB/HE. 'DAB' is the DAB stained cell area and 'HE' is the hematoxylin stained cell area. Later on in this text the word 'fraction' will also be mentioned as 'number'.

3. Statistics

Data analysis and data visualisation was performed using R as statistical software (R version 3.1.0, the R foundation for statistical computing). Statistical significance of the presence of TF on thrombocytes between the cIIMHA and the healthy group, was tested using analysis of variance of a simple linear regression using the lm function (R Core Team, 2014).

4. Results

4.1. Patient selection

All seven cIIMHA dogs had a positive Coombs test along with a Ht < 0.30 L/L at the time of presentation, four dogs also had an increased osmotic red cell fragility, six dogs showed spherocytosis. No underlying disorder that could have provided a trigger for the development of the cIIMHA was identified in any of the patients. Clinical signs and blood results on the first hospitalization day are shown in Table 1.

4.2. Thrombocyte parameters

In cIIMHA patients (day 1–10) the MPM and MPV were both statistical significant ($P < 0.001$) increased and the MPC was statistical significant ($P < 0.001$) decreased, compared with the healthy group (Fig. 1).

4.3. Thrombocyte isolation

By use of the hematology analyzer (ADVIA 2120i Hematology Analyzer, Siemens healthcare) the number of thrombocytes within the thrombocyte fractions was enumerated (*cIIMHA group*: mean 78.3 10e9/L, SD 54.2, range 15–235, n = 29; *healthy group*: 85.89 10e9/L, SD 47.5, range 25–192, n = 9)) and the sample purity was calculated (*cIIMHA group*: mean 99.55%, SD 0.94, range 95.6–100, n = 29; *healthy group*: 99.84%, SD 0.19, range 99.6–100, n = 9). A sample smear confirmed highly pure thrombocyte isolates.

4.4. Immunocytochemistry

The thrombocytes of cIIMHA dogs and healthy dogs stained TF-positive (Fig. 2A and B). Both the isotype control and the slide without primary antibody showed no TF-positive staining. The leukocytes (mainly monocytes but also neutrophils) served as a positive control, since both cells are known to express TF protein (Mälarstig and Siegbahn, 2007; Maugeri et al., 2006; Rivers et al., 1975; Setty et al., 2012). Indeed, the immunocytochemistry of these cells showed TF-positive staining (Fig. 2C). As expected, the isotype control slide and the slide without primary antibody (negative control) showed no TF-

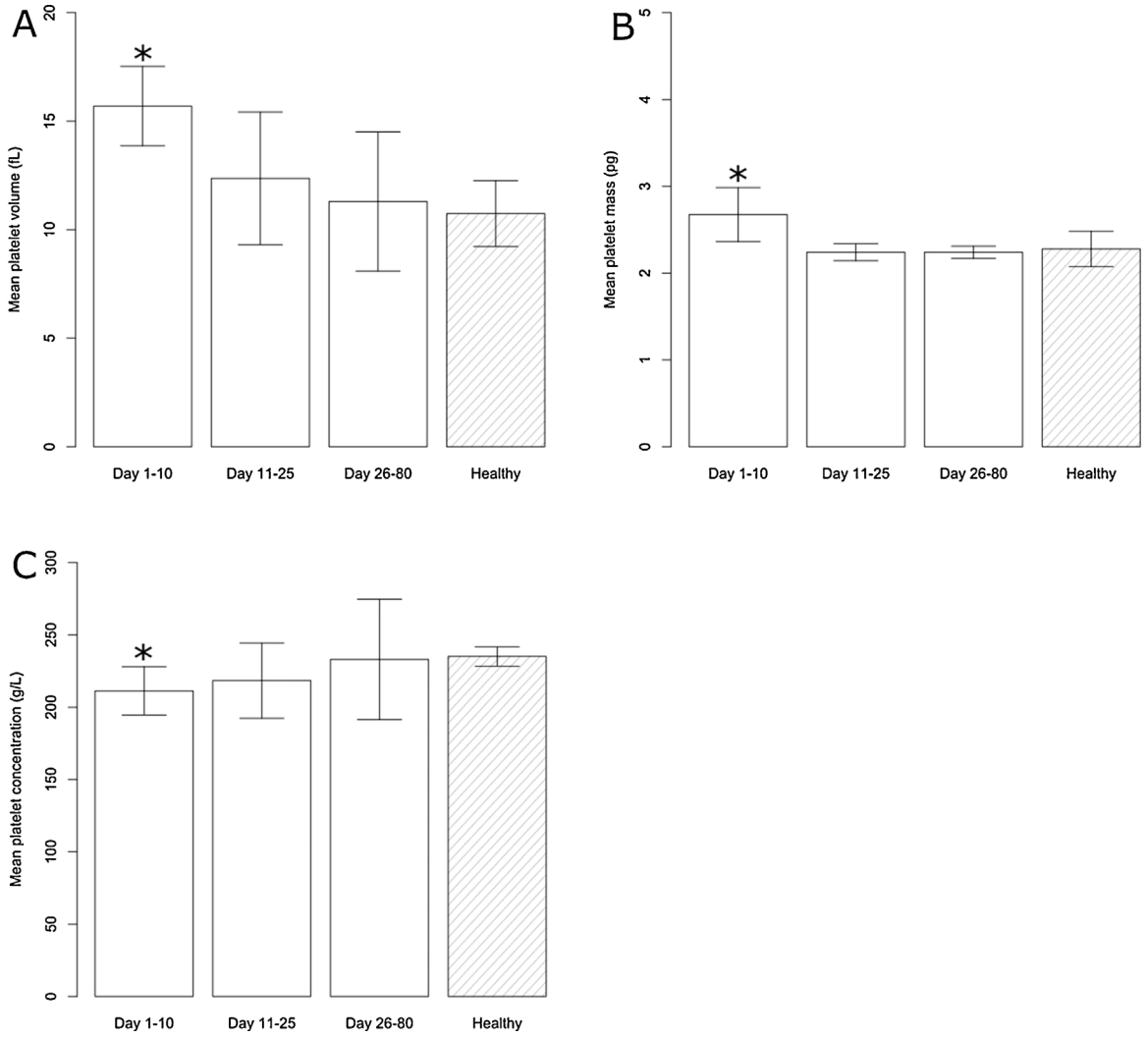

Fig. 1. MPV, MPM and MPC values during hospitalization and follow-up of cIIMHA patients compared with healthy dogs.
Platelet parameters are measured by use of a hematology analyzer (ADVIA 2120i Hematology Analyzer, Siemens healthcare). The cIIMHA patients are followed during time and the healthy dogs served as reference. A Statistical significant (*) increase of MPV between groups 'cIIMHA day 1–10′ (mean 15.7, SD 1.8, range 12.3–19.1, n = 28) and 'healthy' (mean 10.7, SD 1.5, range 8.7–13.1, n = 9). B Statistical significant (*) increase of MPM between groups 'cIIMHA day 1–10′ (mean 2.7, SD 0.3, range 1.8–3.2, n = 28) and 'healthy' (mean 2.5, SD 0.2, range 2.0–2.6, n = 9). C Statistical significant (*) decrease of MPC between groups 'cIIMHA day 1–10′ (mean 211, SD 16.7, range 181 − 238, n = 28) and 'healthy' (mean 235, SD 6.7, range 226 − 245, n = 9). * = $p < 0.001$, *n* = number of blood samples, white bar = cIIMHA dogs, shading bar = healthy dogs.

positive staining. The erythrocytes that served as a negative control showed TF-negative staining, confirming TF absence and specificity of the assay (Fig. 2D).

The relative number of TF positive thrombocytes of cIIMHA dogs was statistically significant higher compared to healthy dogs at hospitalization day ($P < 0.0001$), as shown by normalisation of the TF quantity with 'colour deconvolution' (Fig. 3.). The fraction TF positive cIIMHA thrombocytes decreased towards healthy reference values at approximately 3 weeks after the diagnosis is made (Fig. 4.).

5. Discussion

Our results show that thrombocyte membranes in general express TF, but that more thrombocytes from cIIMHA dogs express TF. This could explain the hypercoagulability of thrombocytes and therefore the increased risk of developing TE and DIC in cIIMHA. In human studies the presence of TF protein in thrombocytes has been shown as well (Brambilla et al., 2008; Camera et al., 2003; Camera et al., 2016; Müller et al., 2003; Panes et al., 2007; Siddiqui et al., 2002; Vignoli et al., 2013; Zillmann et al., 2001). It appears that the number of TF-positive thrombocytes in cIIMHA dogs remains high during the first weeks of the disease (up to 5 weeks after hospitalization). This finding may contribute to the increased risk of TE and DIC found in cIIMHA, specifically within the first two weeks of the disease (Piek, 2011).

A role for TF production by thrombocytes was suggested previously based on high TF gene expression in cIIMHA which coincided with elevated MPV, MPM and decreased MPC (Piek et al., 2011). Statistically significant increase of both MPM and MPV and decrease in MPC was present in the current study during the first 10 days after the diagnosis, and has been associated with an increase in platelet production rate, increased haemostatic capacity, and, respectively, thrombocyte activation (Jackson and Carter, 1993; Karpatkin et al., 1978; Moritz et al., 2005; Piek, 2011). In conclusion, the increase of TF positive thrombocytes and TF gene expression in cIIMHA, together with a decrease of MPC and an increase of both MPV and MPM, suggest a role for thrombocytes in TF production, and may explain the increased risk of developing TE/DIC in the beginning of the disease.

In humans, activated thrombocytes are capable of releasing functionally active TF (Brambilla et al., 2015; Siddiqui et al., 2002). The current study shows that cIIMHA patients carry thrombocytes that are statistically significant more TF positive, compared to healthy dogs. There are some human and mouse studies that argue that monocytes

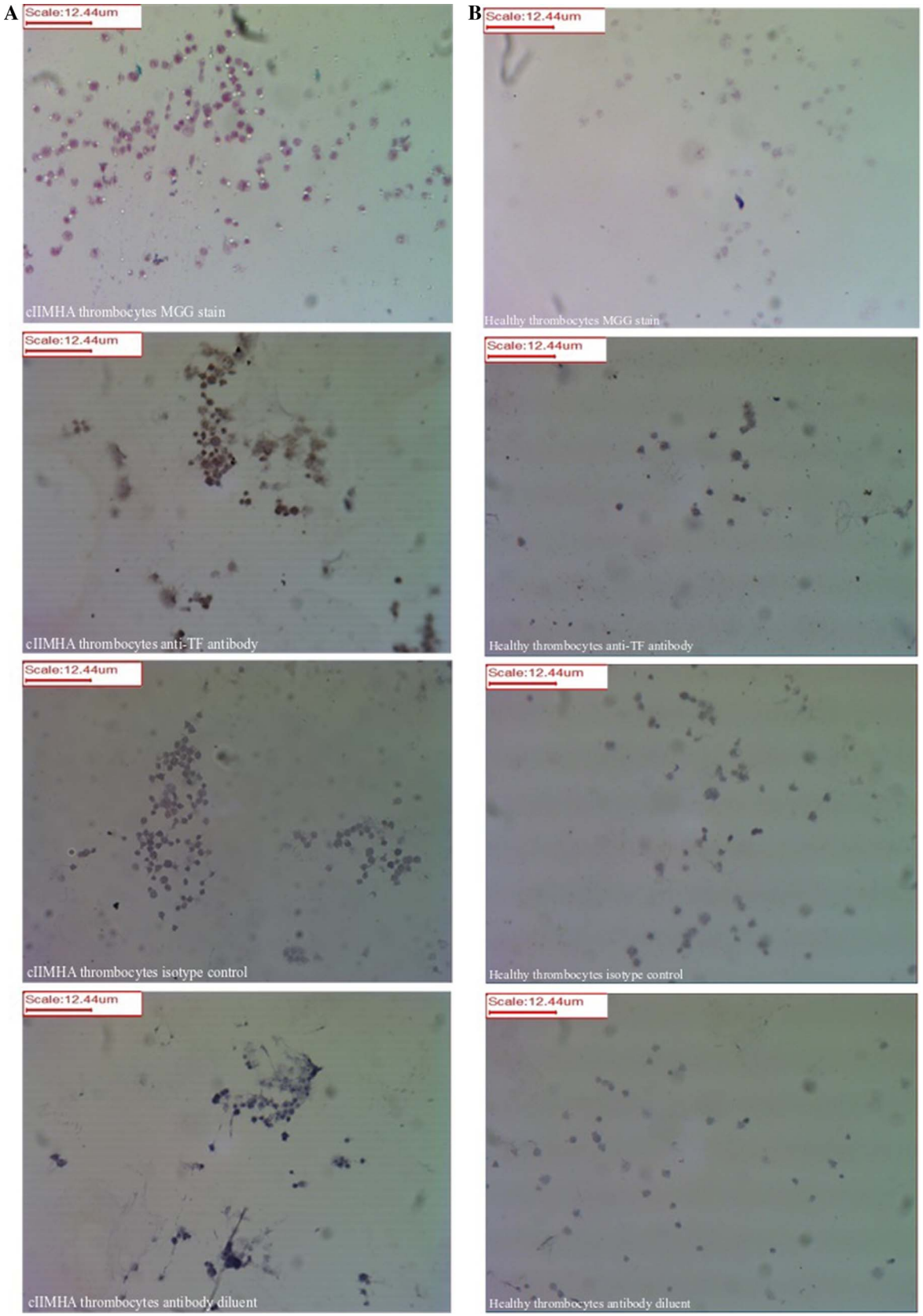

Fig. 2. A selection of ICC results of TF staining with DAB.
Thrombocytes of a cIIMHA dog at hospitalization day (A), thrombocytes of a healthy dog (B), monocytes as positive control (C) and erythrocytes as negative control (D). The upper photos show the slides stained with the May Grünwald Giemsa (MGG) stain; the second photos show the slides incubated with anti-TF antibody; the third photos show the slides incubated with the isotype antibody; the lower photos show the slides incubated with antibody diluent. The MGG stain was used to check cell integrity and cell distribution. A Thrombocytes of a cIIMHA dog at hospitalization day show a TF positive thrombocyte fraction of 0.99 (upper photo). B Thrombocytes of a healthy dog show a TF positive thrombocyte fraction of 0.46 (upper photo). C Monocytes served as a positive control and as expected, they stained TF positive. D Erythrocytes served as a negative control and as expected, they stained TF negative. Original microscope photo magnification: 250x. Scale bar: 12.44 µm.

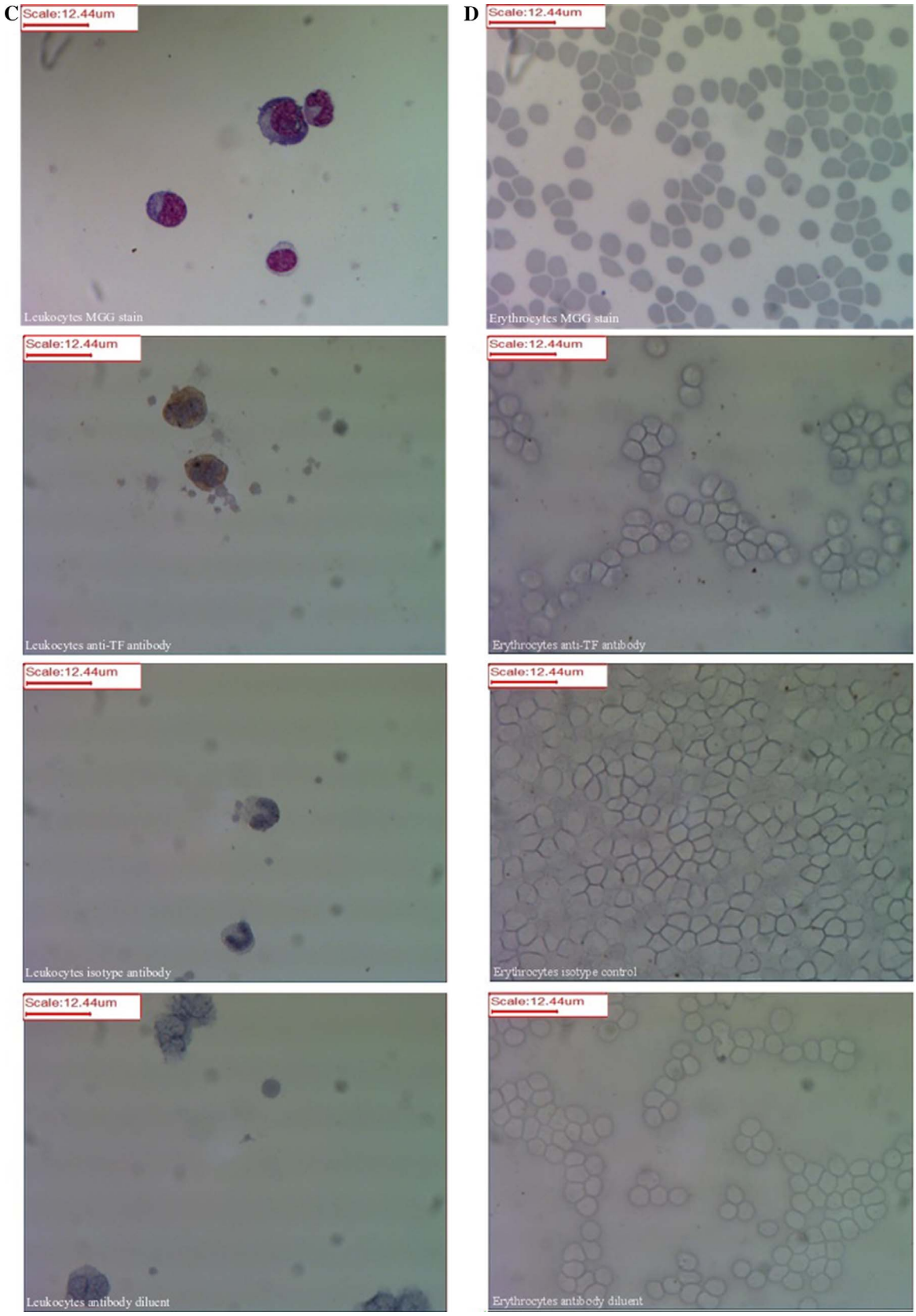

Fig. 2. (*continued*)

donate TF to thrombocytes by microvesicles (Giesen et al., 1999; Rauch et al., 2000; Sovershaev et al., 2012) or that thrombocytes may have inherited TF protein from the megakaryocyte (Brambilla et al., 2015). There are also suggestions from human studies that thrombocytes even inherit TF mRNA from megakaryocytes and therefore may capable of producing and releasing their own functionally active TF protein

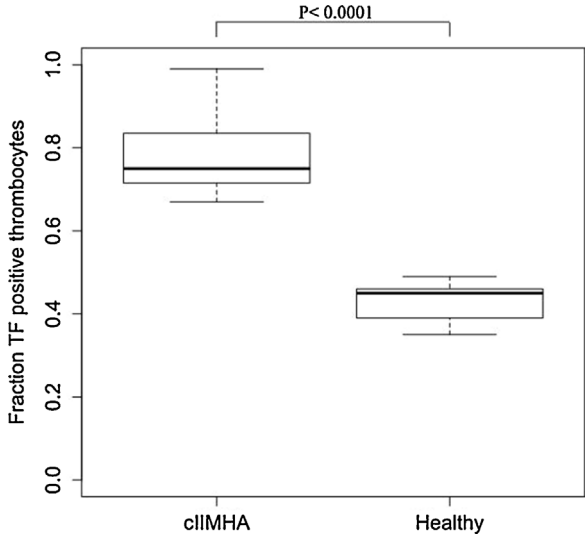

Fig. 3. Normalised TF quantity.
Normalised fractions of TF positive thrombocyte membranes from cIIMHA patients (n = 7) on first hospitalization day compared with the healthy dogs (n = 9). Thrombocyte membranes of cIIMHA dogs stained statistically significant ($p < 0.0001$) more TF positive (SD 0.10; range 0.68–0.99), when compared to the healthy dogs (SD 0.04; range 0.35–0.49). The boxes represent the two middle quartiles with medians. The whiskers delineate the range. *ImageJ* was used to obtain normalised results.

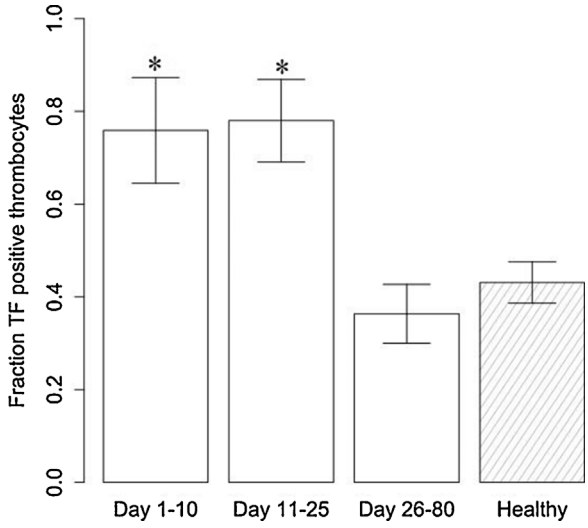

Fig. 4. A bar graph showing the normalised fractions of TF positive thrombocyte membranes of the cIIMHA and healthy group during time. The decrease of the fraction of TF positive cIIMHA thrombocyte membranes is shown during hospitalization (*day 1–10*; mean = 0.79, range = 0.53 − 0.99, n = 24) and during follow-up appointments (*day 11 − 25*; mean = 0.78, range = 0.68–0.85, n = 3 & *day 26 −80*; mean = 0.38, range = 0.29–0.40, n = 3). The healthy dog group (*healthy;* mean = 0.44, range = 0.35–0.49, n = 9) served as reference values. There is a statistical significant difference (*) between de cIIMHA (‘*day 1–10*’ and ‘*day 11–25*’) and the healthy group of $P < 0.05$. In the cIIMHA group the healthy reference values are reached at approximately 3 weeks, up to 11 weeks, after the diagnosis is made. So, there is a wide range in decrease rate until approaching the healthy reference values. *n* = number of blood samples, white bar = cIIMHA dogs, shading bar = healthy dogs.

(Brambilla et al., 2015; Siddiqui et al., 2002). Despite the fact that the exact origin of the thrombocyte associated TF protein in this study is still unknown, it is an interesting finding that warrants further research since it might explain the development of hypercoagulability in the initial phase of cIIMHA that is associated with high mortality.

In our ICC study, the anti-TF antibody visualised the presence of TF protein on the cIIMHA thrombocytes. This TF presence was confirmed by using an isotype control antibody. This isotype antibody tests the specificity of the used anti-TF antibody to TF protein. An internal control for the whole ICC procedure was also used by incubating the third slide (of a series) only with primary antibody diluent. The ICC results of all the cIIMHA dogs showed indeed that the isotype control antibody slides and the slides incubated with the antibody diluent, stained negative to TF protein, whilst the slides incubated with the anti-TF antibody stained positive to TF. An extra control in our ICC procedure was provided by use of erythrocytes, which are cells known to be negative for the presence of TF, and monocytes/neutrophils, which are cells known to be positive for the presence of TF. As expected, the erythrocytes stained negative and the monocytes/neutrophils stained positive. Altogether this also confirmed the specificity of the anti-TF antibody to TF, and proves the presence of TF protein on thrombocytes of cIIMHA dogs.

P-selectin functions as a cell adhesion molecule and plays a role in thrombus formation by promoting thrombocyte recruitment and aggregation (Fenty et al., 2011; Scott-Moncrieff et al., 2001; Weiss and Brazzell, 2006). Increased expression of P-selectin may be an alternative explanation for the increased risk of either developing thromboembolic disease or DIC in cIIMHA patients Visualisation of p-selectin expression, next to TF expression was not within the scope of this study, but it might be an interesting follow up to this current study.

Despite the fact that both monocytes and neutrophils are known to be TF positive, we have chosen to pool these two cell fractions. This ensured in cell rich and thus easy to read cytospins for the ICC procedure. The fact that these samples were contaminated with thrombocytes did not interfere with the ICC results, because quantitative analysis of TF protein was scored on thrombocytes only. We cannot exclude, however, that the presence of TF protein on thrombocytes is due to the shedding of micro-vesicles by blood borne cells such as monocytes/neutrophils that fuse with the thrombocyte membrane (Giesen et al., 1999; Rauch et al., 2000).

Surprisingly, we also detected, albeit low, TF expression in the healthy dogs. This may have been due to trauma induced by jugular venipuncture, or alternatively, due to the in vitro handling of the cells. However, it is clear from the significant difference between the two groups that cIIMHA dogs have significantly more TF positive thrombocytes when compared to healthy dogs and the number of these TF positive thrombocytes in cIIMHA dogs remains high during the first two weeks, up to five weeks, after the diagnosis is made. These findings may well explain the increased risk of developing TE and DIC specifically within the first two weeks of the disease.

In conclusion, it is conceivable that TF plays a role in the development of TE and DIC and therefore in survival of cIIMHA patients. Anti-thrombotics such as ‘anti-tissue factor antibody’, which has been shown positive antithrombotic effects in mouse models (Kirchhofer et al., 2000; Peng et al., 2007) may be considered as an additional therapeutic option. Until then, more evident information about the role and origin of TF in cIIMHA is warranted.

Conflict of interest statement

There are no known conflicts of interest associated with this publication. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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